Expression of the c-myc Protein Is Down-Regulated at the Terminal Stages During In Vitro Differentiation of B-Type Chronic Lymphocytic Leukemia Cells

By Lars-Gunnar Larsson, Marina Schena, Mats Carlsson, Jan Sällström, and Kenneth Nilsson

The translocated c-myc oncogene in Burkitt’s lymphoma (BL) and murine plasmacytoma (MPC) has been proposed to be expressed at a stage of differentiation at which the gene is normally silent, resulting in a continuous proliferation and an inhibited terminal differentiation. To determine whether c-myc is differentially expressed at the various stages of the differentiation pathway, we used B-type chronic lymphocytic leukemia (B-CLL) cells, representing resting B lymphocytes, inducible to proliferation and/or differentiation in vitro. The c-myc protein, and Ig λ-light chain and PCA-1 antigen as markers of B-cell maturation, were analyzed in single, morphologically defined cells by immunocytochemical double-staining. The proliferation of individual cells was determined by ³H-thymidine incorporation and by analysis of Ki-67 anti-
gen expression. The results show that the level of c-myc expression correlates to the stage of differentiation and to the proliferative activity. Uninduced resting cells did not express c-myc. The c-myc protein was observed in the highest amount at the proliferative B-lymphoblast stage of maturation and was reduced in plasmablasts and undetectable in plasma cells. The results suggest that maturation of B cells into nonproliferative, terminally differentiated plasma cells is associated with a downregulated c-myc expression and thus support the view that the deregulated c-myc gene in BL and MPC is expressed at an inappropriate stage of maturation and thereby inhibits terminal differentiation.

MOST HEMATOPOIETIC tumors represent clonal expansions of cells arrested at a preterminal stage of differentiation. Chromosomal aberrations probably play a role in deregulation of the genetic programs controlling growth and differentiation. In B-cell tumors, translocations involving the c-myc and Ig loci are common features in Burkitt’s lymphoma (BL), murine plasmacytoma (MPC), and rat immunocytoma and also occur in acute B-lymphoblastic leukemia (B-ALL). Similar translocations of c-myc, involving the T-cell receptor loci, have been reported in T-ALL. The crucial role of deregulated c-myc expression in malignant transformation of B cells is also suggested by the retroviral integration into the c-myc locus demonstrated in avian and murine lymphomas and by the high incidence of B lymphomas in transgenic mice expressing a Eμ/c-myc transgene.

Although the precise function of the nuclear myc oncprotein is unknown, observations made during recent years suggest that it is involved in the control of growth and/or differentiation. In vitro differentiation of hematopoietic cell lines, eg, the murine erythroleukemia (MEL) and the human HL-60 and U-937, is associated with a downregulation of c-myc expression. The significance of this reduction was further suggested by the observations that terminal differentiation is inhibited in MEL and U-937 cells constitutively expressing a transferred c- or v-myc gene. In BL and MPC, the normal c-myc allele is transcriptionally silent, indicating that the translocated myc oncogene may be expressed at a stage of B-cell differentiation at which c-myc expression normally is nonpermissive. Little is known, however, about c-myc regulation during B-cell differentiation.

Selected B-type chronic lymphocytic leukemia (B-CLL) tumors may be used as model systems for studies of differentiation-associated c-myc expression because B-CLL clones represent resting (G₀) B lymphocytes inducible to activation, proliferation, and differentiation closely resembling that of normal B cells. We previously showed that 12-O-tetradecanoyl-phorbol-13-acetate (TPA) induction of B-CLL cells results in a transition of the cells from G₀ to G₁, an increase in RNA and protein synthesis, an orderly expression of B-cell activation and differentiation surface antigens, an increase in the ratio secretory:membrane Ig µ-heavy chain mRNA, a morphologic transition of the cells into lymphoblasts and plasmablasts and secretion of IgM. The process is accompanied by a dramatic increase in expression of c-myc mRNA and protein. This increase contrasts with the observed downregulation of c-myc during differentiation of MEL, HL-60, and U-937 cells, and suggests that B-cell differentiation is compatible with c-myc expression. Induction of differentiation in B-CLL cells by TPA gives rise to a heterogeneous population of cells representing different stages of maturation, however, and only a few percent of the cells will differentiate terminally into cells with a plasma cell morphology.

To determine whether c-myc expression is preferentially confined to certain stages of B-cell differentiation, we studied the expression of c-myc protein in individual B-CLL cells by immunocytochemical techniques. We used four different protocols for induction of a selected B-CLL clone, 183²²: (a) Staphylococcus aureus Cowan strain 1 (SAC) + B-cell stimulatory factor (BSF-MP6) + tumor necrosis factor-α (TNF-α), resulting in poor differentiation and moderate DNA synthesis; (b) SAC + BSF-MP6 + interleukin-2 (IL-2), leading to moderate differentiation and very high DNA synthesis; (c) TPA, inducing moderate differentiation and no DNA synthesis; and (d) TPA + BSF-MP6 + IL-4, resulting in an efficient differentiation and moderate DNA synthesis. The results suggest that c-myc expression in...
B-CLL cells induced by the different protocols is related to the proliferative activity and to the stage of maturation. The c-myc protein is not detectable in resting, unstimulated B-CLL cells, is expressed at a high level at the proliferative lymphoblast stage of differentiation, is reduced at the plasmablast stage, and again is not detectable at the terminal, nonproliferative plasma cell stage of differentiation.

MATERIALS AND METHODS

Cell culture and reagents. Leukemic, monoclonal (IgM) lymphocytes were obtained from a B-CLL patient (I83) and isolated by Ficoll-Paque gradient centrifugation. The phenotype of the I83 B-CLL cells has been detailed previously.23 I83 cells were cultured in RPMI 1640 medium supplemented with 10% newborn calf serum, 2 mmol/L glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL gentamicin. Heat-inactivated and formalin-fixed SAC particles were used at a final concentration of 0.1%. TPA (Sigma, St Louis, MO) was used at a concentration of 1.6 x 10⁻⁷ mol/L. BSF-MP6, which is necessary for induction of efficient DNA synthesis and differentiation in I83 B-CLL cells,25,26 was obtained in serum-free supernatants from a T-cell hybridoma (MP6)9 and used in concentration of 25% vol/vol. The major active component in BSF-MP6 was recently identified as thiorodoxin.27 Human recombinant IL-2 (specific activity 7 x 10⁴ U/mg) was purchased from Amgen (Amersham, England) and used at a concentration of 100 U/mL. Human recombinant IL-4 (specific activity 10⁹ U/mg) was purchased from Genzyme (Boston, MA) and used at a concentration of 100 U/mL. Human recombinant TNF-α (specific activity 6 x 10⁷ U/mg) was a gift from Dr G. R. Adolph, Ernst-Boehringer Institute, Vienna, Austria, and used at a concentration of 100 ng/mL.

Induction of differentiation. I83 cells, at a density of 2 x 10⁶ cells/mL, were exposed to TPA, SAC + BSF-MP6 + TNF-α, SAC + BSF-MP6 + IL-2 and TPA + BSF-MP6 + IL-4, respectively, for 6 days. When SAC was included in the stimulation protocol, the cells were exposed to SAC for 2 days, followed by BSF-MP6 + TNF-α or BSF-MP6 + IL-2 as previously described.25 The morphologic differentiation was studied on May-Grünwald-Giemsa-stained cytospin slide preparations by light microscopy. A minimum of 400 cells were counted. The functional differentiation was determined by measuring the amount of secreted IgM in supernatants using an enzyme-linked immunosorbent assay (ELISA) technique.24

³H-Thymidine (³H-TdR)-labeling. DNA synthesis was determined by incubating cells in triplicate 0.2-mL cultures with 1 μCi of ³H-TdR (specific activity 6.7 Ci/mmol, Dupont, Boston, MA) for the last 24 hours of a 6-day incubation period. The cells were harvested on a cell harvester (Skatron, Liebyen, Norway), and the incorporated radioactivity was determined by a liquid scintillation counter (Beckman, Irvine, CA). For autoradiography, cytospin slides were fixed in methanol/acetic acid (3:1) or 1% paraformaldehyde (PFA) and overlaid with Kodak (Rochester, NY) nuclear track emulsion, stored for 1 week at 4°C, and then processed with Kodak LX-24 x-ray developer.

Acridine orange (AO) staining. For assessment of RNA and DNA content, the cells were stained by AO according to Darzinskiewicz28 as detailed previously.29

Immunofluorescence and cell sorting. The cells were washed phosphate-buffered saline and 2% fetal calf serum labeled on ice for 30 minutes with monoclonal antibodies (MoAbs) PCA-1 (Coulter Immunology, Hialeah, FL). As a second-layer antibody, an FITC-conjugated goat F(ab')₂ antimouse Ig (Janssen Biochemicals, Beerse, Belgium) was used at a final dilution of 1:80. The cells were washed twice and fixed on ice in 1% PFA in PBS. The cells were subsequently separated into PCA-1⁺ and PCA-1⁻ populations by cell sorting using a FACStar flow cytometer (Beckton Dickinson, Mountain View, CA).

RNA isolation and analysis. Total cellular RNA was isolated by the LiCl/area procedure.29 Denatured total RNA samples (15 μg/well) were fractionated on a 1% formaldehyde-containing agarose gel, transferred to a nitrocellulose filter, hybridized with a ³²P-labeled probe, and washed under stringent conditions as previously described.30 A 1.3-kb EcoRI/Clal fragment prepared from a human c-myc clone31 and spanning the third exon of the human c-myc gene was used as a probe. The fragment was ³²P-labeled by random priming (Multiprime DNA labeling kit, Amersham) to a specific activity > 10⁶ cpm/μg DNA. Autoradiograms were analyzed by densitometric scanning (Shimadzu CS-930, Kyoto, Japan).

Immunocytochemistry. The cells were cytospin-dried, fixed for 30 minutes, and then fixed in 1% PFA for 30 minutes. After three washes in PBS, the slides were incubated for 30 minutes with normal goat serum, washed, and subsequently stained or double-stained for 1 hour with specific antibodies. The antibodies used were rabbit anti-human c-myc antiserum (diluted 1:400; provided by Dr R. R. Watt of Smith, Kline and French Laboratories (Philadelphia, PA), Mouse anti-KI-67 (Dakopatts, Copenhagen, Denmark), diluted 1:10 and anti-human k-chain MoAbs (Becton Dickinson) (diluted 1:40). For blocking experiments, the anti-c-myc antiserum was incubated for 3 hours at room temperature with 1 μg recombinant human c-myc protein31 (provided by Dr R Watt) per microliter of antiserum before the cells were labeled. Goat anti-rabbit Ig antibodies and swine anti-mouse Ig antibodies, respectively, were used as a second layer. The subsequent procedures were used Vectastain peroxidase ABC reagents (Vector Laboratories, Burlingame, CA) for the c-myc and Ki-67 stainings and APAAP (Dakopatts, Copenhagen, Denmark) for the k-chain staining. The peroxidase activity was visualized by 3-amin-9-ethylcarbazol and the alkaline phosphatase activity by naphtol AS-MX phosphate/fast blue. A minimum of 400 cells was counted.

RESULTS

Induction of proliferative and nonproliferative differentiation of B-CLL cells. To obtain populations of cells at different levels of differentiation and proliferation, I83 B-CLL cells were induced by SAC + BSF-MP6 + TNF-α, SAC + BSF-MP6 + IL-2, TPA, and TPA + BSF-MP6 + IL-4, respectively, for 6 days. As shown in Table 1, the functional maturation, as measured by the amount of IgM secreted, was poor in SAC + BSF-MP6 + TNF-α induced cells (162 ng/mL), intermediate in SAC + BSF-MP6 + IL-2 induced cells (420 to 560 ng/mL), and high (2,600 ng/mL) in TPA induced cells. This was also reflected by the percentage of plasmablasts/mature plasma cells, which was only 3% with the TNF-α protocol and 35% with the IL-4 protocol (Table 1). The DNA synthesis, as measured by ³H-TdR incorporation, was only minimal in TPA-induced cells, intermediate (73 x 10⁴ cpm) in SAC + BSF-MP6 + TNF-α-stimulated cells, high in TPA + BSF-MP6 + IL-4-induced cells (305 x 10⁴ cpm), and very high (665 x 10⁴ cpm) in cells exposed to SAC + BSF-MP6 + IL-2. Table 1 also shows that the majority of TPA-induced cells (65%) entered the cell cycle but were blocked in G₀/G₁, as determined by AO staining, whereas a substantial fraction of the SAC + BSF-MP6 + IL-2⁻ and TPA + BSF-MP6 + IL-4⁻ population was arrested in G₀/G₁. An autoradiogram taken from TPA-induced cells is shown in Figure 2. The percentage of cells in each phase of the cell cycle is given in Table 1.
stimulated cells traversed the cell cycle and expressed the proliferation-associated nuclear antigen Ki-67.35
c-myc mRNA expression during proliferative and nonproliferative differentiation of B-CLL cells at the population level. To determine whether the expression of c-myc mRNA was correlated to the level of DNA synthesis, differentiation, or both, RNA purified from the different cultures was subjected to RNA gel blot analysis and hybridized to a 32P-labeled c-myc probe. As shown in Fig 1, c-myc transcripts were hardly detectable in uninduced cells. A drastic increase in c-myc mRNA expression was observed 4 hours after TPA induction. The level of c-myc mRNA in the nonproliferative TPA–induced cells at 6 days after induction was comparable with that observed in the highly proliferative SAC + BSF-MP6 + IL-2–stimulated cells and 20-fold higher than in uninduced cells. The lowest expression of c-myc, one-sixth of the level of that in TPA–induced cells, was observed in the TPA + BSF-MP6 + IL-4–stimulated cells, ie, the protocol most efficiently inducing differentiated cells despite their high level of DNA synthesis. Accurate loading of the RNA samples was verified by EtBr-staining (data not shown).

Expression of c-myc protein at the single cell level. As evident from Table 1, in vitro differentiation of B-CLL cells gives rise to a heterogeneous population of cells representing different stages of activation and maturation. The information obtained by a RNA gel blot analysis therefore will represent only the average expression of c-myc mRNA at the population level. To determine whether the expression of c-myc was confined to certain subpopulations of cells, we used immunocytochemical methods to study expression of the c-myc protein at the single cell level, using an anti-human c-myc antiserum.

In contrast to the c-myc– uninduced cells, most of the stimulated cells expressed an intense nuclear staining after 4-hour TPA exposure (Fig 2A and B). TPA-induced cells labeled with antiserum preblocked by recombinant human c-myc protein were negative, confirming the specificity of the reaction (Fig 2C). After 6 days of TPA treatment, 90% of the cells were still c-myc–, although the intensity in staining among different cells was heterogeneous (Table 2). Most of the cells, however, showed a weaker staining than at 4 hours after induction. In addition, after stimulation by SAC + BSF-MP6 + TNF-α and SAC + BSF-MP6 + IL-2, the majority of the cells (95%) expressed the c-myc protein. In contrast, a larger fraction of TPA + BSF-MP6 + IL-4–induced cells were c-myc– or weakly c-myc+, in agreement with the results obtained by Northern blot analysis. Most of the cells within this fraction did not resemble small resting lymphocytes, however.

Differences in c-myc expression at different stages of maturation. To define the different cell types more precisely with respect to stage of maturation, we combined morphologic evaluation with immunocytochemical double-labeling of the cells using anti-myc and anti-λ-chain antibodies. The results are shown in Tables 2 and 3. The highest frequency of strongly λ+ cells (60%) was observed after stimulation with TPA + BSF-MP6 + IL-4, which is in agreement with the high level of Ig secretion and the high number of mature cells among these cells (Table 1).

Several cell populations could be defined on the basis of the combined staining of c-myc and cytoplasmic λ-chain. First, regardless of the induction protocol used, approximately 5% of the cells remained c-myc– and λ-chain– (myc–λ–). These cells had the morphology of uninduced, small resting lymphocytes. Second, a larger group of cells were λ-chain+ and usually weak in c-myc staining (myc–λ+). These cells had the morphology of small lymphocytes (Fig 2D) but often had a slightly larger cytoplasm than uninduced cells. This was the dominating cell type (44%) when the least efficient differentiation inducing protocol (SAC + BSF-MP6 + TNF-α) was used, but represented a minority of cells (15%) stimulated by TPA + BSF-MP6 + IL-4, the protocol most efficiently
among the TPA + BSF-MP6 + IL-4-stimulated cells were found in these three populations. A similar distribution was observed among cells stimulated with the other inducers (data not shown).

The functionally more mature cells, defined by an intense \( \lambda \)-chain staining, can be grouped into three populations with respect to \( c\text{-}myc \) protein expression (Table 2). First, the fraction of those with an intense \( c\text{-}myc \) staining (\( myc^{++\lambda^+} \)) was dominated by B-lymphoblasts (Table 3, Fig 2E and F). The frequency of these cells was elevated after induction by SAC + BSF-MP6 + IL-2, the protocol most efficiently inducing proliferation. Only a minority of the cells within this population resembled plasmablasts. Second, the fraction of weakly \( c\text{-}myc \)’ cells (\( myc^{+\lambda^{++}} \)) mainly consisted of plasmablasts (85%) (Table 3, Fig 2G) and some lymphoblasts. Third, the fraction of \( c\text{-}myc \)’ cells (\( myc^{+\lambda^{++}} \)) comprised small to middle-sized cells resembling plasma cells (60%) and larger plasmablasts. The \( c\text{-}myc \) plasma cell-like cells usually exhibited a very intense \( \lambda \)-chain staining (Fig 2H). The \( c\text{-}myc^{+\lambda^{++}} \) and the \( c\text{-}myc^{+\lambda^{++}} \) fractions were essentially absent (3%) in cultures stimulated with the least efficient maturation-inducing protocol (SAC + BSF-MP6 + TNF-\( \alpha \)), but represented a substantial fraction (36%) of the cells stimulated by the most efficient maturation inducing protocol (TPA + BSF-MP6 + IL-4) (Table 2).

Increased number of \( c\text{-}myc^- \) cells in populations enriched for mature cells. To clarify further whether the strongly \( \lambda \)-chain/"c\text{-}myc" population indeed consisted of the most differentiated cells, we enriched the mature populations from the TPA + BSF-MP6 + IL-4-induced cells using antibodies to a marker present on differentiated B cells, the PCA-1 antigen.\(^{23}\) PCA-1 was expressed on 10% to 15% of TPA + BSF-MP6 + IL-4-induced cells and absent from cells induced by the three other protocols.\(^{21}\) We separated PCA-1-labeled, PFA-prefixed TPA + BSF-MP6 + IL-4-induced cells into PCA-1’ and PCA-1’ populations by cell sorting. The two populations were subsequently double-stained for \( c\text{-}myc \) and \( \lambda \)-chain proteins. The results are presented in Table 4.

The frequency of \( \lambda \)-chain’ cells was increased in the PCA-1’ population. The fraction of cells with intense staining was also increased (data not shown). The most striking difference was, however, that the relative number of strongly \( \lambda \)-chain positive \( c\text{-}myc^- \) cells was increased fourfold in the PCA-1’ population.

DNA synthesis and Ki-67 expression at the single cell level after induced differentiation of B-CLL cells. Because TPA + BSF-MP6 + IL-4 not only induces efficient maturation of 183 cells but also a high level of DNA synthesis and an equivalent high number of Ki-67’ cells (Table 1), we wished to compare the distribution of proliferating cells among the different cell types with that of \( c\text{-}myc \) expressing cells. Therefore, cells were induced by TPA + BSF-MP6 + IL-4 for 6 days and labeled with \(^{3}H\)-TdR during the last 2 or 24 hours of culture and subsequently stained for cytoplasmic \( \lambda \)-chain or by May-Grünwald-Giemsa. In parallel, cells were double-labeled for Ki-67 and \( \lambda \)-chain. Table 5 shows that 25% of the induced cells were Ki-67 positive and 16%
Cytospin slides were subsequently analyzed for expression of c-myc and the cytoplasmic \(\lambda\)-chain by immunocytochemical double-staining, using antihuman c-myc antiserum and monoclonal anti-\(\lambda\)-chain antibodies, as detailed in the Materials and Methods section. A minimum of 400 cells was counted. Representative data from one of at least two separate experiments are presented. Negative (-): weakly positive (+); strongly positive (++); very strongly positive (+++); mixed population of weakly and strongly positive cells (+/++). Whether the increased c-myc expression in stimulated B-CLL cells was confined to certain subpopulations of cells was unclear, however. To address this question, we used different protocols of induction resulting in more or less differentiated populations under proliferative or nonproliferative conditions and analyzed expression of the c-myc protein in individual cells by immunocytochemical methods. The results suggest that the c-myc protein is differently expressed in different populations of cells and correlates with the stage of maturation. Thus, with respect to c-myc expression at least five different populations were observed: (a) c-myc- small lymphocytes, (b) c-myc+ activated lymphocytes, (c) strongly c-myc+ lymphoblasts, (d) weakly c-myc+ plasmablasts, and (e) c-myc+ plasmablasts and plasma cells.

Our results thus suggest that expression of c-myc protein is downregulated at the most terminal stages of B-CLL differentiation. This suggestion is based on the following observations. First, the c-myc- and strongly \(\lambda\)-chain+ (myc+ \(\lambda^{++}\)) cells resemble plasma cells and plasmablasts by the shape and size, the low nuclear/cytoplasmic ratio, and the relatively small nucleus, positioned eccentrically in the prominent cytoplasm. Cytoplasmic and nuclear structures, eg, the Golgi complex, nucleoli and the chromatin are difficult to evaluate by this technique, however. Second, myc \(\lambda^{+++}\) are the most intensely anti-\(\lambda\)-chain-stained cells observed. Third, the fraction of the myc \(\lambda^{+++}\) cells correlates in size with the efficiency of the induction protocols to

**DISCUSSION**

Our previous studies showed that TPA-induced nonproliferative differentiation of B-CLL cells was associated with a drastically increased c-myc expression, which contrasted with the reduced c-myc expression observed during in vitro differentiation of several myeloid and erythroid cell lines. and 33% of the cells had synthesized DNA during a 2- and 24-hour \(^{3}\text{H}-\text{TdR}\) pulse, respectively, as determined by autoradiography. Morphologic examination showed that most of both Ki-67+ and replicating cells were B lymphoblasts. Among the total number of lymphoblasts, 65% were Ki-67 positive and 40% synthesized DNA during a 2-hour pulse as compared with only 10% to 15% among the lymphocytes and plasmablasts. No Ki-67+ or replicating cells with plasma cell morphology were observed; however, 46% of the plasmablasts and 13% of the plasma cells had synthesized DNA during the last 24 hours of a 6-day stimulation.

**Table 2. Expression of c-myc Protein and \(\lambda\)-Chain in Different IB3 Populations After 6 Day Induced Differentiation (percentage of cells)**

<table>
<thead>
<tr>
<th>Population</th>
<th>myc- (\lambda)</th>
<th>myc+ (\lambda)</th>
<th>myc+ (\lambda^{++})</th>
<th>myc+ (\lambda^{+++})</th>
<th>myc- (\lambda^{++})</th>
<th>myc- (\lambda^{+++})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninduced</td>
<td>99</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TPA (4 h)</td>
<td>11</td>
<td>89</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SAC + BSF-MP6 + TNF-(\alpha)</td>
<td>5</td>
<td>44</td>
<td>38</td>
<td>10</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>SAC + BSF-MP6 + IL-2</td>
<td>4</td>
<td>28</td>
<td>31</td>
<td>29</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>TPA</td>
<td>5</td>
<td>25</td>
<td>36</td>
<td>17</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>TPA + BSF-MP6 + IL-4</td>
<td>5</td>
<td>15</td>
<td>22</td>
<td>22</td>
<td>21</td>
<td>15</td>
</tr>
</tbody>
</table>

The analysis was performed as described in the footnote to Table 2.

**Table 3. Distribution of Morphologically Defined Cell Types Among \(\text{myc/}\lambda\)-Stained Populations After 6-Day Induction by TPA + BSF-MP6 + IL-4 (percentage of cells)**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Lymphocytes</th>
<th>Lymphoblasts</th>
<th>Plasma blasts</th>
<th>Plasma Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>myc- (\lambda)</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>myc++ (\lambda)</td>
<td>96</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>myc++ (\lambda^{++})</td>
<td>86</td>
<td>14</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>myc++ (\lambda^{+++})</td>
<td>12</td>
<td>73</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>myc++ (\lambda^{+++})</td>
<td>4</td>
<td>11</td>
<td>63</td>
<td>2</td>
</tr>
<tr>
<td>myc++ (\lambda^{+++})</td>
<td>0</td>
<td>1</td>
<td>37</td>
<td>62</td>
</tr>
</tbody>
</table>

The analysis was performed as described in the footnote to Table 2.

**Table 4. Separation of TPA + BSF-MP6 + IL-4-Induced Cells Into PCA-1 and PCA-1' Populations (percentage of cells expressing c-myc protein and \(\lambda\)-chain)**

<table>
<thead>
<tr>
<th>Population</th>
<th>myc- (\lambda)</th>
<th>myc+ (\lambda)</th>
<th>myc+ (\lambda^{++})</th>
<th>myc+ (\lambda^{+++})</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCA-1</td>
<td>6</td>
<td>18</td>
<td>65</td>
<td>11</td>
</tr>
<tr>
<td>PCA-1'</td>
<td>3</td>
<td>5</td>
<td>44</td>
<td>48</td>
</tr>
</tbody>
</table>

Cells were labeled with anti-PCA-1 MoAbs, fixed in 1% PFA, separated into PCA-1- and PCA-1+ populations by cell sorting using FACS. Cytospin slides were subsequently analyzed for expression of c-myc protein and \(\lambda\)-chain by immunocytochemical techniques. The intensity of the staining is not quantified.

**Table 5. Distribution of Ki-67+ and \(^{3}\text{H}-\text{TdR}\)-Incorporating Cells Among Morphologically Defined Cell Types After Six-Day Induction by TPA + BSF-MP6 + IL-4 (percentage of cells)**

<table>
<thead>
<tr>
<th>Proliferation Markers</th>
<th>Lymphocytes</th>
<th>Lymphoblasts</th>
<th>Plasma blasts</th>
<th>Plasma Cells</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67+</td>
<td>7 (16)*</td>
<td>14 (44)</td>
<td>4 (11)</td>
<td>0 (0)</td>
<td>25</td>
</tr>
<tr>
<td>3(^{3}\text{H}-\text{TdR}) incorporation</td>
<td>4 (9)</td>
<td>9 (41)</td>
<td>3 (9)</td>
<td>0 (0)</td>
<td>16</td>
</tr>
<tr>
<td>24-hour pulse</td>
<td>6 (14)</td>
<td>16 (73)</td>
<td>11 (46)</td>
<td>1 (13)</td>
<td>33</td>
</tr>
</tbody>
</table>

The cells were labeled with 1 \(\mu\text{Ci}\) \(^{3}\text{H}-\text{TdR}\) for the last 2 or 24 hours of the 6-day culture and were fixed and subjected to autoradiography as described in the Materials and Methods section. The cells were stained by May-Grünwald-Giemsa or by anti-\(\lambda\)-chain antibodies. Expression of Ki-67 was analyzed by immunocytochemical methods.

*Percentage of positive cells in relation to the total number of cells of a defined cell type is shown in parentheses.
induce differentiation, representing 1% of the cells in SAC + BSF-MP6 + TNF-α-stimulated cultures and 14% when cells were stimulated with TPA + BSF-MP6 + IL-4. Fourth, cell sorting of TPA + BSF-MP6 + IL-4-induced cells into PCA-1+ and PCA-1− populations showed a fourfold increase in the number of c-myc−λ+++ cells in the PCA-1+ population.

c-myc expression evidently is compatible with Ig production because the two proteins are co-expressed in a large fraction of cells. These cells are also likely to secrete IgM because 70% of TPA + BSF-MP6 + IL-4-induced cells are secretory, as shown previously by Carlsson et al using the ELISPOT technique. The very high level of Ig production observed in the most mature cells is associated with downregulated c-myc expression, however.

Our results suggest that c-myc expression is not directly correlated to the level of DNA synthesis when related to the cell population as a whole, because the level of c-myc mRNA expression was sixfold higher in the nonproliferative TPA-induced cells as compared with the proliferating TPA + BSF-MP6 + IL-4-stimulated cells. The c-myc expressing TPA-induced cells are competent to enter S-phase, however, provided that appropriate growth factors are present, as evidenced by addition of BSF-MP6 or BSF-MP6 + IL-4.22,24 BSF-MP6 + IL-4 alone does not stimulate DNA synthesis.24 Thus, c-myc expression itself is not sufficient to transfer B cells into S-phase but may be required, as previously suggested.20,23 Our results indicate a correlation between c-myc expression and proliferation because most of the strongly c-myc+ TPA + BSF-MP6 + IL-4-stimulated cells are found within the subpopulation (lymphoblasts) that predominantly expresses Ki-67 and synthesizes DNA. Similar observations were made by Holte et al22 of normal and malignant B cells.

Based on the present results, which are summarized in Fig 3, we suggest that the differentiation process is separable into two stages. The first stage is associated with an induced c-myc expression and is characterized by transition of the cells from G0 to G1, acquisition of responsiveness to growth/differentiation factors, blast transformation, transition through the cell cycle and initiation of Ig production. The second stage, during which c-myc expression is downregulated, begins during the plasma blast stage and is characterized by a high level of Ig production and secretion, condensation of the chromatin, and exit from the cell cycle.

The increased relative frequency of plasmablasts and plasma cells that had incorporated [3H]-TdR during the last 24 hours as compared with a 2-hour pulse after 6-day stimulation with TPA + BSF-MP6 + IL-4 (Table 5) indicates a flow of cells through the cycling lymphoblast compartment into the mature noncycling plasmablast/plasma cell compartment, as shown in Fig 4. The results also show that differentiation with concomitant proliferation is far more efficient in producing mature plasma cells.

**Fig 3.** Expression of c-myc protein in B-CLL cells in relation to differentiation and growth. Induced c-myc expression correlates with activation, blast transformation, and proliferation (stage I). c-myc is down-regulated during terminal differentiation into nonproliferative plasma cells (stage II).

**Fig 4.** Hypothetical view of the cell cycle progression during induced differentiation of B-CLL cells. Self-renewal is favored at the expense of differentiation among the highly c-myc-expressing SAC + BSF-MP6 + IL-2-stimulated cells and vice versa in TPA + BSF-MP6 + IL-4-induced cells expressing lower c-myc levels.
than is the nonproliferative TPA protocol. Anderson and Melchers suggested that proliferation is even necessary to obtain terminally differentiated plasma cells. The IL-4 protocol thus induces both growth and an efficient differentiation. The IL-2 protocol, on the other hand, appears to maintain the cells within the cell cycle to a greater extent and thus favors self-renewal at the expense of maturation (Fig 4). The high level of c-myc expression induced by IL-2 may affect the balance between self-renewal and differentiation in these cells. Such observations have been made in transgenic mice expressing a Eμ/myc gene that results in an accumulation of proliferating immature B cells.

The present results are thus in agreement with the observations in MEL,11 HL-60,14 and U-93715,16 that c-myc expression is downregulated during the terminal part of differentiation, associated with growth arrest. The results also suggest that the translocated c-myc gene in BL and MPC is expressed at a stage of differentiation, corresponding roughly to the resting B cell and plasmablast stage, respectively, during which c-myc expression in B-CLL is downregulated. Kakkis and Calame suggested that the transcriptional repression of the normal c-myc allele observed in MPC is mediated by a regulatory DNA-binding protein that is specifically active in plasmacytomas but not in B cells at an earlier stage of differentiation. One may hypothesize that the deregulated c-myc expression in BL and MPC cells, similarly to the situation in c- or v-myc expressing MEL,17 U-937 cells,16 avian macrophages,19 and murine preadipocytes,20 make them unable to enter a resting state (G0/G1) and to complete the differentiation process.

ACKNOWLEDGMENT

We thank Klaas van Haeringen for valuable help in analysis of Ki-67 expression and of 3[H]-TdR incorporation in individual cells. We also thank Drs Christer Sundström, Thomas Töterman, Ulf Pettersson, and Georg Klein for helpful discussions, and Eva Harrysson for help in manuscript preparation. We thank Dr Rose-Mary Watt for the donation of anti-human c-myc antiserum and recombinant human c-myc protein.

REFERENCES

10. Steffen D: Proviruses are adjacent to c-myc in some murine leukemia virus-induced lymphomas. Proc Natl Acad Sci USA 81:2097, 1984
gize to induce B-chronic lymphocytic leukemia cells to simultaneous immunoglobulin secretion and DNA synthesis. Leukemia 2:734, 1988


40. Freytag SO: Enforced expression of the c-myc oncogene inhibits cell differentiation by precluding entry into a distinct predifferentiation state in G0/G1. Mol Cell Biol 8:1614, 1988
Expression of the c-myc protein is down-regulated at the terminal stages during in vitro differentiation of B-type chronic lymphocytic leukemia cells

LG Larsson, M Schena, M Carlsson, J Sallstrom and K Nilsson